NITROGEN DISTRIBUTION IN GLOBIN.

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In endeavoring to isolate any single component from a naturally occurring mixture of proteins, one is almost certain to be embarrassed by the lack of some safe yet convenient criterion which shall guarantee the completeness of the separation. The writers lately experienced this difficulty in attempting to obtain preparations of globin which should be demonstrably uncontaminated by the other proteins of the blood. They had barely encountered the problem when its solution seemed to be offered. Fürth and Lieben (1) reported in 1920 the entire absence of the tryptophane radicle from the molecule of hemoglobin. Here appeared to be the desired criterion. The other blood proteins are known to be fairly rich in tryptophane, so that the problem apparently resolved itself into one of purifying hemoglobin, or its globin component, until it gave a negative glyoxylic reaction.

We have already reported elsewhere the failure of the expectations thus raised (2). It has been found that not even five recrystallizations of hemoglobin made any appreciable difference in the intensity of the glyoxylic reaction as given either by the pigment itself or by the globin prepared from it. Not only is this so, but the method of tryptophane estimation based by Fürth and Nobel (3) upon the Voisenet reaction—the very method the use of which had led Fürth and Lieben to conclude that hemoglobin is free from tryptophane—indicated in our hands the presence in globin of 2.6 per cent of that amino-acid. This proportion was not in the least diminished by successive purifications. We were forced to the conclusion—quite in accord with earlier belief (4, 5)—that tryptophane is an integral part of the globin molecule; and could only suppose that Fürth and Lieben had been misled by the use of globin solutions altogether too dilute to give the Voisenet reaction.

This and other experiences with the tryptophane method of Fürth and Nobel led us to doubt seriously the reliability of quantitative data obtained by its application. When, therefore, just as we completed our work with it, Folin and Looney (6) described another and apparently better method of determination, a method based upon a different color reaction and capable moreover of convenient combination with a quantitative procedure for tyrosine, it seemed to us worth while to review the problem again. With the aid of this newer method we have now determined the tryptophane and tyrosine content of two series of globin preparations, and have, we believe, settled fairly decisively the proportion of these amino-acids yielded by the pure protein. We have also taken occasion to determine by the method of Van Slyke the general distribution of nitrogen in the globin molecule.

Meanwhile, our original conclusion has been independently confirmed in Fürth's own laboratory. Kiyotaki (7), still using the method of Fürth and Nobel, but observing certain precautions neglected by Fürth and Lieben, estimates the tryptophane content of globin at the rather high figure of 3.6 per cent. He has also determined the tyrosine content, which he places at 3.5 to 4 per cent. Since in each determination his result as well as his method is different from ours, the presentation of our data would still seem to be worth while.

Globin Preparations.

First Series.—A large quantity of crystalline oxyhemoglobin was obtained from horse blood by the method of Zinoffsky (8). The preparation was recrystallized four times. Of each of the first four crops of crystals a portion was reserved. From each reserved portion, and from the whole of the fifth and final crop, globin was separated by a slight modification of the method of Robertson (9). Five successive specimens of globin, of presumably increasing purity, were thus obtained. The first and fourth were somewhat contaminated with hematin; but the second, third, and fifth were nearly colorless.

Second Series.—The second series of globin preparations differed from the first in two respects. The original solution of hemoglobin, obtained from the laked corpuscular mass, was treated with alumina cream according to the method of Marshall and Welker (10);

and the number of recrystallizations was reduced to two. The series included, therefore, only three specimens. Of these the first and second were rather pigmented; the third was practically white.

The nitrogen content of the globin preparations, determined by the method of Kjeldahl, varied between 16.76 and 16.93 per cent. The average found was 16.86, which agrees with the value 16.89 reported by Schulz (11). The variations, such as they were, were quite irregular, and in themselves gave no evidence of increasing purity in successive fractions.

Determinations of Tryptophane and Tyrosine.

1 gm. lots of the eight preparations of globin, each of which had been dried for 48 hours *in vacuo* over sulfuric acid, were hydrolyzed with baryta, and assayed for tryptophane and tyrosine by the method of Folin and Looney (6). Each determination was carried out on duplicate samples of the product of hydrolysis, the volume taken—4 to 5 cc.—being always such as would yield color depths approximately equal to those of the standards.

The results are reported in Table I, in which is shown also for each case the indicated molecular ratio of tyrosine to tryptophane.

TABLE I.

Source of globin.		Dry globin.		Molecular ratio $\frac{\text{Tyrosine}}{\text{Tryptophane}}$.
		Tryptophane.	Tyrosine.	
		per cent	*per cent*	
First series.	First crystallization	1.93	3.23	1.9
	Second "	1.99	3.57	2.0
	Third "	2.51	3.97	1.8
	Fourth "	2.49	3.96	1.8
	Fifth "	2.48	4.13	1.9
Second series.	First "	2.56	4.10	1.8
	Second "	2.61	4.64	2.0
	Third "	2.62	4.63	2.0

Discussion of the Tryptophane and Tyrosine Determinations.

It is at once apparent from the data that no *diminution* of tryptophane content is effected by successive attempts at purifica-

tion. Whether the *increase* observed in the earlier samples of each series, and the higher maximum shown by the second, have a real significance is not quite certain. The differences seem, especially in the first series, to be rather beyond the experimental error of the method; but the fact that they are paralleled by nearly proportional changes in tyrosine content makes them difficult to explain by simple contamination of the first fractions with at any rate the *serum* proteins. Serum globulin (ox) contains, according to Folin and Looney, 6.7 per cent of tyrosine and 2.28 per cent of tryptophane. A highly purified specimen of serum albumin (horse), to which we applied Folin and Looney's procedure, yielded values of 4.63 per cent for tyrosine and 1.79 per cent for tryptophane. The gradual removal of serum proteins would therefore be expected to raise the tryptophane content of the preparations, but to depress their tyrosine content. Actually the proportion of *each* amino-acid is seen to increase regularly to a maximum, while the molecular ratio remains nearly constant. Possibly an admixture of stroma proteins, regarding the composition of which we know next to nothing, may be responsible for the low tyrosine content of the early fractions.

Taking the analytical results at their face value, it would seem that to obtain a globin of constant composition by simple recrystallization of oxyhemoglobin, three crystallizations at least are necessary; whereas, when a preliminary treatment with alumina cream is employed, two will suffice. This is a confirmation of the claims made (10) for the efficacy of alumina cream in purifying oxyhemoglobin from other colloids.

There is, it must be admitted, a discrepancy between the two series in respect to the ultimate values, especially for tyrosine, to which they rise. While we cannot at present offer an adequate explanation, we feel that the second series deserves the greater confidence, and would therefore put the tryptophane yield of pure globin at 2.61 per cent and its tyrosine at 4.63. This would attribute to each amino-acid the same proportion—2.12 per cent —of the total globin nitrogen.

At these values we get a molecular ratio of tryptophane to tyrosine that is exactly 1:2. On the assumption that this implies the presence in the globin molecule of two tryptophane and four tyrosine radicles, we may then calculate the molecular

weight of globin to be from 15,630 to 15,640. This agrees surprisingly with the 15,274 deduced by Osborne from the sulfur content (12).

The tryptophane value of 2.6 per cent, yielded by the method of Folin and Looney, is identical with that which we formerly obtained (2) by the method of Fürth and Nobel; but the latter in the hands of Kiyotaki (7) gave much higher figures—from 3.1 to 4.0, with an average of 3.6. A higher value by the Fürth-Nobel procedure is the general rule (6), and it may be that the identity of our two results is the chance outcome of the conditions under which the earlier determinations were made. Kiyotaki shows, what our own experience had already suggested, that the result of a Fürth-Nobel determination is greatly dependent upon the concentration of the protein analyzed. We suspect that it is affected by still other factors. We have observed, for instance, that the Fürth-Nobel procedure gives a far deeper and at the same time redder color when applied to glycyl-tryptophane than when applied to an equivalent quantity of free tryptophane;[1] and that the few instances where the Folin-Looney result is not decidedly lower than the other—casein (6), serum albumin (1.8 per cent by Folin and Looney, 1.4 by Fürth and Nobel (7)), and globin (if the comparison is limited to our own determinations)—include only proteins that are free from glycocoll. These observations are too limited in number to be decisive; but they certainly suggest that the Voisenet color reaction is affected, both as to hue and as to intensity, by the mode of combination of tryptophane in the protein molecule. This suspicion is not calculated to increase one's confidence in any method of which it forms the basis.

The tyrosine content of globin was determined by Kiyotaki in two ways; (1) by a bromine titration, and (2) by a colorimetric application of the diazo reaction. Neither method gave very consistent results, the percentage found varied from 2.2 to 5.5. The figure 3.5 to 4.0 is proposed as a probable approximation.

From his data Kiyotaki calculates that if globin has a molecular weight of about 15,000, it should contain 3 molecules each

[1] Proteins also give a redder color than pure tryptophane, a fact already commented upon by Lüscher (Lüscher, E., *Biochem. J.*, 1922, xvi, 556), who produces, moreover, fairly conclusive evidence that values obtained by the Fürth-Nobel method are 30 to 60 per cent too high.

of tyrosine and of tryptophane. We feel that the greater consistency of our own results, together with the pains taken to prove the purity of our globin, make our estimate the more probably correct one.

General Distribution of Nitrogen in Globin.

The preparation of globin used for the Van Slyke analysis had been obtained from oxyhemoglobin, twice crystallized after treatment with alumina cream. It had a light brown color, and included, therefore, doubtless a trace of hematin. Two portions were separately hydrolyzed with 20 per cent hydrochloric acid, the one (designated A in Table II) by heating in a bath of boiling water for 48 hours, the other (B) by boiling over a free flame for 30 hours. Table II shows the results obtained with aliquot portions of each hydrolysate. The original analytical data have been corrected for the solubility of the phosphotungstates of the bases. No correction was applied for cystine, for like Van Slyke in his analysis of hemoglobin (13) we found, even in the much larger quantities with which we were dealing, no evidence of the presence in the base fraction of any appreciable quantity of that amino-acid; the solution absorbed only a trace of bromine in the method of Okuda (14), and a one-fifth aliquot, ignited with the reagent of Denis, yielded less than 1 mg. of $BaSO_4$.

TABLE II.

Distribution of Nitrogen in Globin.

	Nitrogen.		Percentage of total nitrogen.			Calculated from analysis of hemoglobin.
	A	B	A	B	Average.	
	mg.	*mg.*	*per cent*	*per cent*	*per cent*	
Total N taken	375.4	457.0				
Ammonia	19.3	25.6	5.14	5.60	5.37	5.35
Humin	7.0	8.8	1.86	1.93	1.90	1.5
Arginine	30.3	36.4	8.07	7.97	8.0	7.85
Histidine	47.1	58.6	12.55	12.8	12.7	13.0
Lysine	42.4	49.5	11.3	10.8	11.1	11.1
Total bases	119.9	144.5	31.9	31.6	31.8	32.0
" filtrate	225.2	277.0	60.0	60.6	60.3	61.2
Amino N of filtrate	213.8	259.9	57.0	56.9	57.0	58.2
Non-amino N of filtrate	11.4	17.1	3.0	3.7	3.3	3.0
Total N recovered	371.4	455.9	99.0	99.8	99.4	(100.0)

Discussion of Nitrogen Distribution.

Considering the somewhat different conditions of hydrolysis under which the two analyses were conducted the agreement between their results is almost surprisingly good. It may be added that two other analyses, carried through upon entirely different preparations of globin, gave figures differing only within the recognized limits of the method from those presented. Our data stand, moreover, in excellent accord with those obtained by Van Slyke (13) in an analysis of ox hemoglobin. This is shown by the figures in the last column of Table II, where Van Slyke's results have been recalculated for globin upon the assumptions that hematin accounts for 2.1 per cent of the total nitrogen of hemoglobin, and that all this appears in the humin fraction.

The proportion of histidine found may be compared with the estimate of Hanke and Koessler (15), whose colorimetric method indicated the presence in horse hemoglobin of 8.8 per cent histidine, corresponding to 13.8 per cent of the total globin nitrogen.

It is perhaps of some interest to attempt from the analytical data an estimate of the number of basic radicles in the globin molecule. This is made possible by the knowledge already gained that 2.12 per cent of the total nitrogen corresponds to four monoamino-acid (tyrosine) radicles. One amino group, therefore, corresponds to 0.53 per cent of the total globin nitrogen. Dividing the percentages of arginine, histidine,and lysine nitrogen by 4, 3, and 2, respectively, and each quotient by 0.53, we find that globin yields 3.8 molecules of arginine, 8.0 of histidine, and 10.4 of lysine; or, taking the integers to which these numbers approximate, 4, 8, and 10 molecules of arginine, histidine, and lysine, respectively. The exact nitrogen percentages to be expected from such a composition (assuming always the precise correctness of our tyrosine determination) are 8.48 for arginine, 12.72 for histidine, and 10.60 for lysine.

A similar calculation based upon the percentage of filtrate amino nitrogen indicates the presence of 108 monoamino-acid radicles in globin.

While such estimates have no claim to precision, they probably approximate pretty closely to the actual composition of the protein. In the case of the bases they can hardly differ by more than unity from the true number.

SUMMARY.

Analytical data are presented which suggest that the globin molecule yields upon hydrolysis 2 molecules of tryptophane, 4 each of tyrosine and arginine, 8 of histidine, 10 of lysine, and approximately 100 other amino-acid molecules, including dicarboxylic acids.

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